Knockdown of IG20 Gene Expression Renders Thyroid Cancer Cells Susceptible to Apoptosis

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Aim: The aim of the study was to investigate the expression and function of the IG20 gene in thyroid cancer cell survival, proliferation, and apoptosis.

Methods: We determined the expression levels of the major isoforms of IG20 by quantitative RT-PCR in normal and thyroid tumor tissues/cell lines. We evaluated the functional consequence of IG20 knockdown in WRO (follicular carcinoma) and FRO (anaplastic carcinoma) thyroid cancer cell lines by measuring spontaneous, TNFα-related apoptosis-inducing ligand (TRAIL), and TNFα-induced apoptosis.

Results: The IG20 gene expression levels were higher in benign and malignant thyroid tumors and in WRO and FRO cells relative to normal tissues. Predominantly, MADD and DENN-SV isoforms of IG20 gene were expressed. IG20 knockdown resulted in increased spontaneous, TRAIL-, and TNFα-induced apoptosis in WRO, but not FRO, cells. FRO cell resistance to apoptosis is likely due to caspase-8 deficiency.

Conclusion: IG20 knockdown renders WRO cells more susceptible to spontaneous, TRAIL-, and TNFα-induced apoptosis and thus demonstrates the prosurvival function of the IG20 gene in thyroid cancer. These observations, combined with overexpression of IG20 noted in thyroid tumor tissues, may suggest a potential role in thyroid cancer survival and growth and indicate that IG20 may be targeted either alone or in conjunction with TRAIL or TNFα treatment in certain thyroid cancers. (J Clin Endocrinol Metab 94: 1467–1471, 2009)
variants (IG20-SVs), namely IG20pa, MADD/DENN, IG20-SV2 and DENN-SV (3–5). Relative levels of expression of various splice isoforms can profoundly affect cancer cell survival, proliferation, and death (6–8). The MADD and DENN-SV isoforms are constitutively expressed in all tissues and at much higher levels in cancer cells and tissues (3). Using short hairpin RNA (shRNA) that targets exon 15, expressed in all IG20 isoforms, we were able to knock down all isoforms of IG20 in HeLa (cervical cancer) and PA-1 (ovarian carcinoma) cells (9, 10). Knockdown of IG20 resulted in spontaneous as well as enhanced TRAIL (TNFα-related apoptosis-inducing ligand)-induced apoptosis (9, 10). Because one of our earlier studies had shown overexpression of this gene in thyroid tumors and tumor cell lines relative to normal cancer. Our results showed that this gene is expressed at higher levels in thyroid tumors and tumor cell lines relative to normal thyroid tissues, has a prosurvival function in certain thyroid cancer cells, and can confer resistance to TRAIL- and TNFα-induced apoptosis.

Materials and Methods

Human thyroid tissues and cell lines

Twenty thyroid tissues, including seven normal, three follicular adenomas, and five papillary, two follicular, one oncocytic, one anaplastic, and one medullary thyroid carcinoma were obtained (Section of Endocrinology and Metabolism, University of Siena, and Department of Pathology, University of Pittsburgh) under institutional review board-approved protocols. A control thyroid RNA was obtained from Clontech (Mountain View, CA).

WRO (follicular carcinoma) and FRO (anaplastic carcinoma) cells were grown at 37°C with 5% CO2 in RPMI 1640 supplemented with 2 mM l-glutamine, 10% fetal bovine serum, penicillin G 100 U/ml, streptomycin 100 μg/ml, and amphotericin B.

Semi-quantitative RT-PCR

Total RNA from cells and tissues was used for first-strand cDNA synthesis (Invitrogen, Carlsbad, CA) followed by 30 cycles of PCR using denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, followed by a final incubation at 72°C for 7 min. Amplified products were separated on a 5% Tris-Borate-EDTA-buffer polyacrylamide gel electrophoresis and stained with ethidium bromide. The PCR involved 5 min hot start at 95°C, followed by denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, followed by a final incubation at 72°C for 37 min. Cells were solubilized in 10% sodium dodecyl sulfate and 0.01 N HCL, and the OD was determined at 550 nm wavelength. The experiment was repeated twice in triplicate.

Apoptosis assay and caspase-8 detection

Five days after transduction, cells were washed in PBS, stained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) (5 μg/ml) for 5 min, and analyzed for condensed chromatin as an indicator of apoptosis. Seventy-two hours after transduction, TNFα (100 ng/ml; R&D Systems, Minneapolis, MN) and TRAIL- (0.1–200 ng/ml; PeproTech, Rocky Hill, NJ) induced apoptosis was determined by fluorescence-activated cell sorting after propidium iodide (PI) staining. For caspase-8 detection, cell lysates were subjected to SDS-PAGE, followed by immunoblotting using an anti-caspase-8 antibody (Cell Signaling Technology, Danvers, MA).

Statistical analysis

Statistical analyses were performed using Student’s t test, and the values are shown as means ± s; a P < 0.05 was considered significant.

Results

Expression of IG20 splice variants in thyroid cancer tissues and cell lines

As determined by quantitative real-time RT-PCR, the mean value of IG20 expression relative to the level of GAPDH in normal tissues (n = 8) was 1.5 ± 0.2. However, this ratio was significantly higher in all thyroid tumors (n = 13) 3.7 ± 0.6 (P = 0.013) (Fig. 1A). Similarly, both WRO and FRO cells expressed much higher levels of IG20 transcripts. All tested thyroid tissues and cells expressed only MADD and DENN-SV isoforms of IG20 (Fig. 1, B and C).

Effect of IG20 knockdown on cell proliferation

Both WRO and FRO cells were transduced with lentivirus encoding either Scr shRNA or Mid shRNA. RNA extracted from treated cells was subjected to RT-PCR using IG20-specific primers. The IG20 transcripts were essentially absent by 72 h in Mid shRNA, but not Scr shRNA, transduced cells (Fig. 1C). The WRO cells transduced with Mid shRNA showed strong inhibition of proliferation relative to the control and the Scr transduced cells. In contrast, the FRO cells did not exhibit a significant reduction in proliferation upon IG20 knockdown (Fig. 1D).
Effect of IG20 knockdown on TRAIL- and TNFα-induced apoptosis

The effect of IG20 knockdown on the survival of thyroid cancer cells was detected by chromatin condensation using Hoechst staining. Although WRO cells showed higher spontaneous apoptosis upon IG20 knockdown relative to controls, FRO cells failed to undergo spontaneous apoptosis. The above results were confirmed by an alternate method using PI staining.

Although WRO cells transduced with Mid shRNA for 5 d showed over 40% spontaneous apoptosis, WRO cells transduced with Scr shRNA or FRO cells transduced with either shRNA failed to show increased apoptosis (data not shown).

Next, we determined the effects of IG20 knockdown on TRAIL- or TNFα-induced apoptosis in WRO and FRO cells. As expected, cotreatment with cycloheximide and TNFα induced apoptosis in both cell types (data not shown). Although WRO
cells were sensitive to a very low concentration of TRAIL (0.1 ng/ml), the FRO cells were highly resistant even when treated with 200 ng/ml of TRAIL. Upon IG20 knockdown, WRO cells became even more sensitive, whereas the FRO cells remained insensitive to TRAIL (Fig. 2A). Similarly, we saw a modest increase in TNFα-induced apoptosis upon IG20 knockdown in WRO cells but not in FRO cells (Fig. 2B).

Both TRAIL- and TNFα-induced activation of the extrinsic apoptotic pathway is initiated by caspase-8 activation. Because we failed to detect significant levels of TRAIL- or TNFα-induced apoptosis in FRO cells, even after IG20 knockdown, we tested for the levels of caspase-8 by Western blotting and found that FRO cells harbored significantly reduced levels of caspase-8 relative to WRO cells (Fig. 2C).

**Discussion**

The IG20 gene is overexpressed in human tumors and cancer cell lines. Physiologically relevant loss-of-function studies using...
shRNA showed that knockdown of IG20-SVs can result in spontaneous apoptosis of cancer cells (9, 10), but not normal cells (4). These studies demonstrated an indispensable role for IG20 in cancer cell survival.

We observed overexpression of the IG20 gene in most of the thyroid tumor tissues and WRO and FRO cells due to selective expression of MADD and DENN-SV isoforms. Upon IG20 knockdown, a significant increase in spontaneous apoptosis and a reduction in cell proliferation were noted in WRO cells, but not in FRO cells. The cell death and reduced proliferation noted in WRO cells were due to spontaneous apoptosis as indicated by nuclear condensation and PI staining (not shown).

Unlike Fas ligand, TRAIL can selectively induce cancer cell apoptosis with little or no effect on normal cells (12–18). Therefore, we investigated the effect of IG20 knockdown on TRAIL treatment. The WRO cells were more susceptible to a relatively low concentration (i.e., 0.1 ng/ml) of TRAIL, and upon IG20 knockdown they became even more sensitive. In contrast, FRO cells remained resistant at 2000-fold higher concentration of TRAIL (200 ng/ml), even when coupled with IG20 knockdown. Similarly, WRO but not FRO cells became more sensitive to TNFα-induced apoptosis upon IG20 knockdown.

Earlier, MADD has been shown to up-regulate TNFα-induced activation of Erk1/2 (19), and more recently we discovered that MADD is required for Grb2 recruitment to TNFR1, which in turn recruits SOS1/2 leading to sequential activation of Ras, Raf, MEKK and Erk1/2. MADD may confer resistance to apoptosis through its essential role in the activation of Erk1/2, which in turn recruits SOS1/2 leading to sequential activation of Ras, Raf, MEKK and Erk1/2. MADD may confer resistance to apoptosis through its essential role in the activation of Erk1/2, which in turn can up-regulate prosurvival proteins (20).

MADD can also act as a negative regulator of caspase-8 activation (10). Therefore, we expected enhanced cell death resulting from increased caspase-8 activation upon IG20 knockdown. Although enhanced cell death was observed in WRO cells, FRO cells remained resistant. Interestingly, FRO cells expressed much lower levels of caspase-8 relative to WRO cells (Fig. 2C), suggesting that this deficiency may have contributed to the resistance of FRO cells to extrinsic pathway-induced apoptosis.

Further studies are required to determine whether TRAIL or TNFα treatment in the presence of MAPK kinase inhibitors or upon reexpression of procaspase-8 will render FRO cells susceptible to apoptosis. The fact that IG20 is expressed at higher levels in thyroid cancers and knockdown of IG20 renders thyroid cancer cells more susceptible to apoptosis suggests that it is a desirable therapeutic target.

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